Remarks

Claims 1-10 and 12-27 are pending, claim 11 having been previously canceled without prejudice. Claims 1-4, 9, 10 and 19-23 are allowed. Claims 5, 6, 24 and 25 are currently amended. Support for addition of the phrase "in a patient" in amended claims 5, 6, 24 and 25 is found in paragraphs [0001] to [0003], [0009], Example 1 (paragraphs [0031-0041]) and paragraph [0101]. Typographical errors have been corrected in one phrase of claim 24. Claims 5, 6, 24 and 25 have also been amended by rearranging the phrases comprising the terms agonist or antagonist to clarify the meaning of the claims. No new matter has been introduced.

Paragraphs [0090], [0091], and [0096] are currently amended. Support for amended paragraphs [0090] and [0091] is found in Table 1b. Support for amended paragraph [0096] is found in Table 1a.

Response to Informalities/Objections

The Examiner has objected to the reference to "Table 2" in Paragraphs [0090] and [0096], asserting that there is no Table 2 in the specification.

Paragraph [0090] has been amended to recite the term "Table 1b" instead of "Table 2." This correction is obvious because peptides VI, VII, VIII, and X, which are the subject of discussion in paragraph [0090], are summarized in Table 1b. Paragraph [0096] has been amended to recite the term "Table 1a" instead of "Table 2." This correction is obvious because peptide IV, which is the subject of discussion of paragraph [0096], is summarized in Table 1a.

The Examiner has objected to the indication in paragraph [0091] that "peptide X (SEQ ID NO:25)" has an alanine/cysteine substitution. The Examiner asserts that "peptide IX (SEQ ID NO:24)" has the alanine/cysteine substitution, not "peptide X (SEQ ID NO:25)." Paragraph [0091] has been amended by correcting the reference to a substitution of cysteine at P10 with alanine to indicate that the substitution occurs in "peptide IX (SEQ ID NO:24)," not in "peptide X (SEQ ID NO:25)." It can be seen in Table 1b and in the Sequence Listing that peptide IX (SEQ ID NO:24) is the only peptide listed that lacks a C-terminal cysteine.

Applicants submit that the objections to paragraphs [0090, 0091, and 0096] are overcome.

Response to rejection of Claims 5-8, 12-18 and 24-27 under 35 U.S.C. § 112, first paragraph, enablement

Claims 5-8, 12-18 and 24-27 stand rejected as allegedly lacking enablement. The Examiner asserts that the specification is enabling for a method of binding a peptide factor as an antagonist or agonist to a laminin receptor in vitro. However, the Examiner alleges that the method comprising administering to a cell culture a composition of a peptide factor comprising amino acid residues 33-42 of murine epidermal growth factor (mEGF), does not reasonably provide enablement for a method of binding a peptide factor as an agonist or antagonist to a laminin receptor in vivo.

The Examiner then alleges at page 4 of the Office Action that claims 5-8, 12-18 and 24-27 encompass a method of binding a peptide factor to a laminin receptor, comprising administering amino acid residues 33-42 of mEGF, wherein the peptide factor has at least one tyrosine of mEGF being substituted with a tyrosine analog or at least one arginine of mEGF being substituted with an arginine analog. The Examiner then alleges that the specification discloses only cursory conclusions without data to support the findings, which state that a peptide factor derived from amino acid residues 33-42 of mEGF can be used as a treatment for angiogenic disease via its binding to the 67 kDa laminin receptor (citing pages 2-3 of the specification). The Examiner alleges that there are no indicia that the present application enables the full scope of binding a peptide factor as an antagonist or agonist to a laminin receptor. The Examiner then states at page 7 of the Office Action that the scope of the claims is broader than the enabling disclosure, that the working examples do not demonstrate the outcome of the treatment, which is unpredictable, that the teaching of the specification is limited, and therefore, there is not proper guidance to carry out further experimentation to assess the in vivo binding effect of the modified peptide factor.

Applicants respectfully disagree and submit that claims 5-8, 12-18, and 24-27 are enabled by the specification, for the reasons set forth below.

A specification which discloses how to make and use a claimed invention is presumed to comply with the first paragraph of 35 U.S.C. § 112, unless there is a reason to doubt the objective truth of the specification. *In re Marzocchi*, 439 F.2d 220, 169 USPQ 367 (CCPA 1971). The initial burden of establishing a basis for denying patentability to a claimed invention therefore rests upon the examiner. *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988); *In re Thorpe*, 777 F.2d 695, 227 USPQ 964 (Fed. Cir. 1985); *In re Piasecki*, 745 F.2d 1468, 223 USPQ 785 (Fed. Cir. 1984). Here, the present specification clearly discloses how to make and use the claimed laminin-receptor binding peptides, and how to use them in vitro and in vivo.

It is well-settled that an applicant need not have actually reduced the invention to practice prior to filing in order to satisfy the enablement requirement under 35 U.S.C. §112, first paragraph. MPEP §2164.02 (citing *Gould v. Quigg*, 822 F.2d 1074 (Fed. Cir. 1987)). Indeed, the invention need not contain a single example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation (*In re Borkowski*, 422 F.2d at 908), and "representative samples are not required by the statute and are not an end in themselves" (*In re Robins*, 429 F.2d 452, 456-57, 166 USPQ 552, 555 (CCPA 1970)). Thus, 35 U.S.C. § 112, first paragraph, enablement does not require any working examples.

The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. MPEP §2164.01 (citing *In re Angstadt*, 537 F.2d 498, 504 (C.C.P.A. 1976)). The fact that experimentation may be complex does not necessarily make it undue if the art typically engages in such experimentation. *Id.* Further, the specification need not disclose what is well known to those skilled in the art and preferably omits that which is well-known to those skilled in the art and is already available to the public. MPEP §2164.05(a) (citing *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991)). Enablement does not require a working example. Experimentation is allowed, so long as it is not undue.

The Examiner cites Nelson et al. (J. Biol. Chem. 1996, 271:26179-26186) at page 5 of the office action as prior art which shows that a laminin-antagonist peptide comprising amino acid residues 33-42 of mEGF interacts with a 67 kDa laminin receptor of breast cancer and endothelial cells. The Examiner then alleges that the general knowledge and level of skill in the

art do not supplement omitted description and that "the specification needs to provide specific teachings on the treating conditions for binding of the modified peptide factors to laminin receptor in vivo and the effects of these peptide factors to be considered enabling for variants." Applicants respectfully point out that at the time the application was filed references other than Nelson had disclosed binding of peptides to laminin receptor and modulation of laminin receptor function in vitro and in vivo. The application merely omits that which is well-known to those skilled in the art or which is not necessary for practicing the invention. The state of the art is more fully discussed below.

References cited in the specification have demonstrated various biological properties and binding of laminin analogues and derivatives to the laminin receptor (see paragraphs [0089-0098], citing Ostheimer et al., 1992, Kawasaki et al., 1994, Scott, 1997, and McKelvey et al., 1991). It was known to those of skill in the art at the time the application was filed that peptides which bind to the laminin receptor in vitro and modulate functions described in the specification, such as cell motility and cell adhesion, would also modulate such functions in vivo. For example, Nakai et al. (Cancer Res. 1992, 52:5395-5399; abstract provided herewith) demonstrated that intraperitoneal injection of the laminin receptor-binding peptide, laminin derived peptide $C(YIGSR)_3$-NH_2, inhibits metastasis of human melanoma cells in mice. Sakamoto et al. (Cancer Res. 1991, 51:903-906; abstract provided herewith), showed that another laminin derived peptide, $Lam\beta1_{925-933}$ (CDPGYIGSR-NH_2), binds the laminin receptor and inhibits tumor growth in vivo. Iwamoto et al. (Science 1987, 238:1132-1134; copy provided herewith) tested a broad range of doses (10-1000 μg/g) of $Lam\beta1_{925-933}$ (CDPGYIGSR-NH_2) and found that it inhibits metastasis in vivo. Iwamoto also synthesized and tested analogues of $Lam\beta1_{925-933}$. It should be noted that $Lam\beta1_{925-933}$ (CDPGYIGSR-NH_2) is the same as SEQ ID NO:1 of the present application.

Nelson et al., cited by the Examiner and discussed above, disclosed that $mEGF_{33-42}$ and the laminin fragment $Lam\beta1_{925-933}$ had similar structures and had similar binding affinities for the laminin receptor, as measured by whole cell receptor binding assays (see abstract). Additionally, Gebarowska et al. (Am. J. Pathol. 2002, 160:307-313; copy provided herewith) demonstrated in an in vivo model of proliferative retinopathy, e.g., retinopathy of prematurity (ROP), that

angiogenesis was inhibited by the laminin receptor binding peptide $mEGF_{33-42}$ at doses of 2-10 μg/g. Although Gebarowska published after the application was filed, it further demonstrates the efficacy of laminin receptor-binding peptides in vivo.

Thus, Nakai, Sakamoto, Iwamoto and Gebarowska, each prepared various laminin receptor-binding peptides such as laminin-derived peptides and EGF-derived peptides, and tested them in vitro as well as in vivo. A correlation exists between peptides binding to the laminin receptor and eliciting an effect in vitro and a therapeutic effect in vivo. For example, when comparing the ability of various laminin receptor-binding peptides to inhibit tumor cell invasion in vitro with their ability to inhibit tumor cell invasion and metastasis in vivo, Iwamoto stated "there was an exact correlation between the inhibitory activities of the various peptides in the in vitro and in vivo assays" (Iwamoto et al., Science 1987, 238:1132-1134; see page 1133, column 3, lines 17-19).

Furthermore, based on the teachings of the specification, one of ordinary skill in the art would be able to identify a variety of modified $mEGF_{33-42}$ peptides as claimed which have the ability to bind to the laminin receptor and modulate its function in vivo. Modifications of $mEGF_{33-42}$ are disclosed in the specification (Tables 1a and 1b; SEQ ID NOS:7 and 16-28) and the effects of such modifications on binding and activity of the listed peptides are described in the examples. For example, the modified peptides were synthesized (see paragraphs [0045-0046]) and tested for their affinity to the laminin receptor and for their effects on cell motility and adhesion, relative to $mEGF_{33-42}$ or $Lam\beta1_{925-933}$ (SEQ ID NO:1) (see Examples 1 and 2 and paragraphs [0044, 0065-0068, and 0073-0082]). The specification discloses that intraperitoneal injection of 10 μg of synthetic $laminin\beta\text{-}1_{925-933}$ (SEQ ID NO:1) can prevent the development of ROP in vivo (see Figs. 1a and 1b, and paragraph [0033]). Also, as described above, others had prepared and tested various modified laminin receptor-binding peptides and demonstrated that receptor binding correlates with both in vitro effects and in vivo therapeutic effects.

The specification also discloses that $mEGF_{33-42}$ mediates its anti-angiogenic effects via the 67 kDa laminin receptor (paragraph [0085]), and demonstrates by alanine scanning that amino acid residues at positions 1, 2, 3, and 6 (SEQ ID NOS:21, 22, 23, and 25) of $mEGF_{33-42}$ are essential for these peptides to bind and to modulate receptor mediated activities, including

cell attachment to laminin and cell motility (paragraphs [0089-0093]). The specification further discloses several other modified $mEGF_{33-42}$ peptides (SEQ ID NOS:16-20, 27, and 28) and describes the effects of different types of amino acid substitutions on laminin receptor-binding peptide activity in the modified peptides (see paragraphs [0094-0100]).

Therefore, a series of in vitro and in vivo assays are provided in the specification or were known in the art which would have allowed one of skill in the art at the time the application was filed to identify peptides as claimed which bind to the 67 kDa laminin receptor, the conditions for their use in vivo, and methods to determine whether the peptides act as agonists or antagonists of receptor function.

Adequate working examples are provided in the specification, even though as described above, no working examples are required. Also, as described above, a variety of in vitro and in vivo assays are provided in the specification for testing the function of laminin receptor-binding peptides. Although no numerical data for the function of the modified $mEGF_{33-42}$ peptides listed in Tables 1a and 1b are provided by way of additional tables or figures, verbal descriptions are given as to the effect of the various amino acid substitutions on the affinity of the modified peptides to laminin receptor, and the effects of the substitutions on the ability of the modified peptides to regulate cell motility/migration and adhesion of cells to laminin (see paragraphs [0085-0100]). For example, it is stated in paragraph [0096], referring to a modified $mEGF_{33-42}$ peptide, that "replacement of arginine (P9) with citrulline (peptide IV) (SEQ ID NO: 19) increased both receptor binding and inhibition of attachment to laminin substrata whilst retaining antagonist migratory response." The other paragraphs in this section provide similar information for other peptides listed in Tables 1a and 1b. Thus, the descriptions provided for functions of the modified $mEGF_{33-42}$ peptides, together with the other teachings of the specification, are more than satisfactory to allow the invention to be practiced. In fact, there is no requirement that all data must be provided in an application. Therefore, the present application provides adequate working examples and guidance to enable one of skill in the art to practice the invention.

As described above, at the time the application was filed it was routine in the art to synthesize many different laminin-receptor binding peptides and subject them to various in vitro and in vivo assays, using varying dosages of the peptides. Because such experimentation was

routine in the art, preparation and testing of laminin receptor-binding peptides as described and claimed in the present application to determine whether they have agonistic or antagonistic activity is not undue. Thus, based on the teachings provided in the specification and on the knowledge available at the time the specification was filed, one of ordinary skill in the art would have been able to practice the invention without additional guidance or undue experimentation.

In sum, Applicants respectfully submit that claims 5-8, 12-18, and 24-27 are supported by the disclosure provided in the specification as filed. Therefore, adequate guidance is provided for a skilled artisan to make and/or use the full scope of the invention in vivo as recited in claims 5-8, 12-18, and 24-27. Given the advanced state of the relevant art, the ample disclosure, and the extensive reduction to practice provided in the specification as filed, claims 5-8, 12-18 and 24-27 are enabled and this requirement of 35 U.S.C. § 112, first paragraph, has been satisfied. Thus, Applicant respectfully requests that the rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Response to rejection of Claims 5-8, 12-18 and 24-27 under 35 U.S.C. § 112, second paragraph, indefiniteness

Claims 5-8, 12-18 and 24-27 stand rejected as allegedly indefinite. The Examiner asserts that the claims recite administration of a medicament, but that the claims do not indicate to what or whom the medicament is administered. The Examiner indicates that claims 7, 8, 13-18, 26 and 27 are included in this rejection because they are dependent claims which do not correct the deficiency from which they depend. Applicants point out that claim 12 also depends from claim 5.

Although not necessarily agreeing with the reasoning of the Examiner, independent claims 5, 6, 24, and 25 have been amended to recite that the medicament is administered "to a patient." As indicated above, this amendment is supported throughout the specification, particularly in Figs. 1a and 1b and Examples 1 and 2. Applicants submit that claims 5-8, 12-18, and 24-27 are no longer indefinite as to what or to whom the medicament is administered.

The Examiner also alleges that claims 5-8, 12-18, and 24-27 are indefinite because they do not indicate how the peptide factors which bind to the laminin receptor can be identified as

antagonists or agonists of the laminin receptor. Although not necessarily agreeing with the reasoning of the Examiner, independent claims 5, 6, 24 and 25 have been amended to clarify that the method comprises antagonizing or agonizing a laminin receptor. Claims 5 and 24 now recite "a method of antagonizing a laminin receptor" instead of a method of binding to a laminin receptor as an antagonist. Claims 6 and 25 now recite "a method of agonizing a laminin receptor" instead of a method of binding to a laminin receptor as an agonist.

Applicants also submit that there is no requirement that the claims recite "how" the claimed peptide factors agonize or antagonize the laminin receptor. One of ordinary skill in the art would have understood how an agonist or an antagonist of the laminin receptor would work, based upon the methods and assays disclosed in the specification and on general knowledge of peptide receptor function available at the time the application was filed. An agonist was defined at the time as "a molecule, such as a drug, an enzyme activator, or a hormone, that enhances the activity of another molecule or receptor site" (page 15, Dictionary of Biochemistry and Molecular Biology, 2d ed., J. Stenesh, ed., John Wiley & Sons, New York, 1989; copy provided herewith). An antagonist was defined as "a molecule, such as a drug, an enzyme inhibitor, or a hormone, that diminishes or prevents the action of another molecule or receptor site" (page 29, Dictionary of Biochemistry and Molecular Biology, 2d ed., J. Stenesh, ed., John Wiley & Sons, New York, 1989; copy provided herewith).

One of skill in the art at the time the application was filed knew that the laminin receptor was involved in the regulation of such functions as angiogenesis, tumor growth, diabetic retinopathy, wound healing, and various angiogenesis related diseases (see paragraphs [0002], [0003], Examples 1 and 2, and Figs 1a and 1b). For example, Nelson et al., cited by the Examiner, disclosed that cell adhesion, cell motility, endothelial cell differentiation, angiogenesis, tumor cell chemotaxis, and tumor cell metastasis potential are all induced or increased by laminin, an agonist for the 67 kDa laminin receptor (page 26179, column 1, para. 2). An antagonist of laminin would have the opposite effect.

In addition, it is demonstrated in Figs. 1a and 1b that synthetic lamininβ-$1_{925\text{-}933}$ (SEQ ID NO:1), a laminin agonist, prevents retinopathy of prematurity (ROP) in vivo. Furthermore, a series of assays is outlined in the application which describes the preparation and effects of

various peptide factor agonists and antagonists of the laminin receptor on cell migration, cell adhesion, cell proliferation, and on the ability of the peptides to bind to the laminin receptor (paragraphs [0061-0100]).

Therefore, at the time the application was filed, a multitude of in vitro and in vivo models and assays for the effect of laminin receptor binding peptides on laminin receptor function were available to those of skill in the art. Thus, one of ordinary skill in the art would understand how a peptide factor can be identified as antagonist or agonist.

Applicants submit that, based on the amendments and reasoning described above, amended claims 5-8, 12-18 and 24-27 are now definite.

Conclusion

Based on the foregoing, all claims are believed to be in condition for allowance. An early and favorable action toward that end is earnestly solicited.

Respectfully submitted,

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resulting from crosslinking of protein with compounds formed by peroxidation of lipids. The pigment is brown colored and exhibits green-yellow fluorescence when activated with long wavelength ultraviolet light. *Aka* ceroid pigment; lipofuscin; senility pigment.

agglutinating antibody AGGLUTININ.

agglutination The clumping of bacterial and other cells that is brought about by an antigen–antibody reaction between the particulate antigens on the cell surface and added antibodies.

agglutinin An antibody that can bind to particulate antigens on the surface of cells to produce an agglutination reaction.

agglutinogen A surface antigen of bacterial and other cells that can induce the formation of agglutinins and can bind to them to produce an agglutination reaction.

aggregate 1. MULTIENZYME SYSTEM. 2. METABOLON (2).

aggregate anaphylaxis An anaphylactic shock that is produced by a single injection of antigen.

aggressin A substance that is produced by a microorganism and that, though not necessarily toxic by itself, promotes the invasiveness of the microorganism in the host; the enzymes hyaluronidase and collagenase are two examples.

aglucone The noncarbohydrate portion of a glucoside.

agonist A molecule, such as a drug, an enzyme activator, or a hormone, that enhances the activity of another molecule or receptor site. A hormone that binds to a receptor in a productive manner, triggering the normal response, is an example. *See also* decamethonium; full agonist; partial agonist.

agranulocyte A white blood cell (leukocyte) that contains few, if any, granules in the cytoplasm.

A/G ratio Albumin/globulin ratio.

Agrobacterium tumefaciens *See* crown gall tumor.

agrobactin A linear siderophore of the phenol–catechol type found in *Agrobacterium tumefaciens*.

AHF Antihemophilic factor.

AHG 1. Antihemophilic globulin. 2. Anti-human globulin.

AIA Anti-immunoglobulin antibodies.

AICAR 5-Aminoimidazole-4-carboxamide ribonucleotide; an intermediate in the biosynthesis of purines.

AICF Autoimmune complement fixation.

AIDS Abbreviation for acquired immunodeficiency syndrome; a severe viral disease, caused by a retrovirus. The virus destroys T lymphocytes of the immune system and infects cells within the central nervous system. The syndrome first occurred among homosexuals and users of intravenous drugs (1981) but has since spread throughout the world. Most infections occur through sexual transmission, use of contaminated needles, and as a result of infected mothers passing the virus to newborns.

AIDS virus One of a group of retroviruses implicated as the cause of acquired immunodeficiency syndrome (AIDS). Various virus isolates appear to be closely related members of the same virus group. They have been designated LAV (lymphadenopathy-associated virus), HTLV-III (human T-cell lymphotropic virus type III), IDAV (immunodeficiency-associated virus), and ARV (AIDS-associated retrovirus). Two compound designations, HTLV-III/LAV and LAV/HTLV-III have also been used. It has been proposed that the AIDS retroviruses be officially designated as human immunodeficiency viruses, abbreviated as HIV. *See also* antigenic drift.

AIP Aldosterone-induced proteins.

air dose The dose of radiation delivered to a specified point in air.

air peak The gas chromatographic peak that is produced when a small amount of air is injected with the sample into the chromatographic column.

Akabori hypothesis The hypothesis that the origin of proteins is based on the polymerization of non-amino acid building blocks to form polyglycine and on the subsequent replacement of the α-hydrogens in polyglycine by various R groups in secondary reactions.

Akabori reaction The formation of an alkamine by the reaction of an aldehyde with the amino group of an amino acid.

Al Aluminum.

Ala 1. Alanine. 2. Alanyl.

alanine An aliphatic nonpolar amino acid; α-alanine occurs in proteins and β-alanine occurs in the peptides anserine and carnosine. *Abbr* Ala; A.

alarmone A signal molecule in bacteria that has a regulatory effect on metabolism by exerting control on many biochemical reactions at once. The action of an alarmon is similar to that of a hormone in multicellular organisms. In bacteria, such regulation may come into play in response to environmental stresses. As an example, amino acid starvation results in the accumulation of the compounds known as magic spots. These are believed to function as alarmones, leading to cessation of protein synthesis and cessation of transcription of rRNA genes.

alarm reaction GENERAL ADAPTATION SYNDROME.

albinism A genetically inherited metabolic

be negative when the flow of water is in the opposite direction.

an mer One of two isomeric carbohydrates (designated α and β) that differ from each other only in the configuration about the anomeric carbon of the ring structure. The α-isomer has the hydrogen at the anomeric carbon above (and the β-isomer has it below) the plane of the ring in a Haworth projection.

anomeric carbon The carbon atom of the carbonyl group in a carbohydrate.

anomeric effect The stereochemical effect in carbohydrate chemistry in which the interaction between the oxygen of the monosaccharide ring and the substituent (—OR; —O—CO—R; or halogen) at the anomeric carbon is such as to favor the maximum separation between the oxygen and the substituent; as a result, the axial substituent, or α-anomer, is favored over the equatorial substituent, or β-anomer. The molecule having an equatorial anomeric substituent is less stable than the one having an axial substituent.

ANOVA Acronym for analysis of variance. *Aka* ANOVAR.

anovar Acronym for analysis of variance.

anoxia HYPOXIA.

anoxybiontic Not capable of using atmospheric (molecular) oxygen for growth. *Aka* anoxybiotic. *See also* anaerobic (2,3).

anserine A dipeptide of β-alanine and methyl histidine that occurs in vertebrate muscle.

antagonism The phenomenon in which the action of one agent is counteracted by the action of another agent that is present at the same time.

antagonist A molecule, such as a drug, an enzyme inhibitor, or a hormone, that diminishes or prevents the action of another molecule or receptor site. *See also* α-bungarotoxin.

ante-iso fatty acid A fatty acid that is branched at the carbon atom preceding the penultimate carbon atom at the hydrocarbon end of the molecule.

antenna molecules Molecules that are not photochemically active and merely serve in the capacity of a large antenna, passing the excitation energy in photosynthesis from one molecule to another until it is trapped by the photochemically active molecules in the reaction center. Antenna molecules constitute the bulk of the photosynthetic pigment molecules. *Aka* antenna chlorophyll.

ante-penultimate carbon The third carbon atom from the end of a chain.

anterior 1. In front of, or in the front part of, a structure. 2. Before, in relation to time.

anthesin FLOWERING HORMONE.

anthocyanidin The aglycone of an anthocyanin.

anthocyanins Water-soluble plant pigments that occur largely in the form of glycosides of an anthocyanidin. Anthocyanins are bioflavonoids. *See also* bioflavonoid.

anthranilic acid *See* chorismic acid.

anthrone reaction A colorimetric reaction for carbohydrates, particularly hexoses, that is based on the production of a green color on treatment of the sample with anthrone.

anthropic principle The principle according to which the presence of life on earth may explain some of the conditions associated with life. It is usually argued that life arose on the earth because circumstances, such as a moderate temperature, were conducive to its existence. According to the anthropic principle, the argument is reversed; it is postulated that the presence of life on earth explains why the latter has a moderate temperature.

anti 1. Referring to a nucleoside conformation in which the base has been rotated around the sugar, using the C—N glycosidic bond as a pivot, so that the sugar is in direct opposition to the base. This represents a sterically less hindered conformation than the syn conformation; in polynucleotides, it leads to the bulky portions of the bases being pointed away from the sugar–phosphate backbone of the chain. 2. Referring to a trans configuration for certain compounds containing double bonds, such as the oximes which contain the group C=N—OH. 3. Referring to the position occupied by two radicals of a stereoisomer in which the radicals are farther apart as opposed to the syn position in which they are closer together. *See also* syn.

antiacrodynia factor VITAMIN B_6.

antiadrenergic *See* alpha blocker; beta block r.

antianemia factor VITAMIN B_{12}.

antiantibody An antibody produced in response to an antigenic determinant of an antibody molecule.

antiauxin A compound that functions as a competitive inhibitor of auxin.

antibacterial agent *See* bactericide; bacteristat.

antiberiberi factor VITAMIN B_1.

antibiosis The association of two organisms in which one produces a substance, such as an antibiotic, or a condition that is harmful to the other.

antibiotic Originally, defined as a compound produced by a microorganism that inhibits the reproduction or causes the destruction of other microorganisms. Now more generally defined as a compound produced by a

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Synthetic Peptides Interacting with the 67-kd Laminin Receptor Can Reduce Retinal Ischemia and Inhibit Hypoxia-Induced Retinal Neovascularization

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The high-affinity 67-kd laminin receptor (67LR) is expressed by proliferating endothelial cells during retinal neovascularization. The role of 67LR has been further examined experimentally by administration of selective 67LR agonists and antagonists in a murine model of proliferative retinopathy. These synthetic 67LR ligands have been previously shown to stimulate or inhibit endothelial cell motility *in vitro* without any direct effect on proliferation. In the present study, a fluorescently labeled 67LR antagonist (EGF_{33-42}) was injected intraperitoneally into mice and its distribution in the retina was assessed by confocal scanning laser microscopy. Within 2 hours this peptide was localized to the retinal vasculature, including preretinal neovascular complexes, and a significant amount had crossed the blood retinal barrier. For up to 24 hours postinjection, the peptide was still present in the retinal vascular walls and, to a lesser extent, in the neural retina. Non-labeled EGF_{33-42} significantly inhibited pre-retinal neovascularization in comparison to controls treated with phosphate-buffered saline or scrambled peptide ($P < 0.0001$). The agonist peptide ($Iam\beta1_{925-933}$) also significantly inhibited proliferative retinopathy; however, it caused a concomitant reduction in retinal ischemia in this model by promoting significant revascularization of the central retina ($P < 0.001$). Thus, 67LR appears to be an important target receptor for the modulation of retinal neovascularization. Agonism of this receptor may be valuable in reducing the hypoxia-stimulated release of angiogenic growth factors which drives retinal angiogenesis. *(Am J Pathol 2002, 160:307–313)*

The inappropriate proliferation of retinal capillaries derived from pr - xisting vessels (retinal neovascularization) is a significant complication of many important ocular conditions such as diabetic retinopathy, branch vein occlusions, and retinopathy of prematurity. Togeth r these conditions constitute major causes of blindness and yet the ability to prevent neovascularization is severely limited and is currently reliant on ablation of functional retina using laser photocoagulation or cryotherapy. The underlying basis for retinal neovascularization and the complexity of the angiogenic stimulus is becoming clearer. Vascular endothelial growth factor (VEGF) and other related angiogenic peptides are now known to have a critical role in initiating and propagating the neovascular response[1] and effective neutralization of these factors is a hopeful avenue for therapeutic intervention. However, this optimism must be tempered by the realization that many such factors are also important promoters of vascular cell survival,[2] therefore putative inhibitory substances would need to be carefully titrated and delivered within defined periods of the proliferative response. An alternative approach to controlling retinal neovascularization is to antagonize adhesion-dependent migration of activated endothelial cells. Agents which can block receptor-mediated interactions of migrating endothelial cells with the extracellular matrix (ECM) would be expected to preferentially target the actively proliferating retinal vessels.[3,4]

Laminin is a major component of vascular basement membranes and is vital for endothelial cell function under physiological conditions.[5] Cellular interaction with laminin α, β, and γ chains is achieved through a range of integrin and non-integrin receptor interactions that coordinate cellular adhesion, spreading, differentiation, and phenotypic stabilization.[6] Among the many laminin-binding proteins, a high-affinity non-integrin laminin receptor which migrates at 67 kd, after posttranslational modification of a ~33-kd precursor protein (designated P40/37LRP),[7,8] has been identified in vascular endothelial cells.[9,10] This receptor (designated 67LR) binds to a cysteine-rich domain of the short arm of laminin $\beta1$.[11]

Tumor cell-associated 67LR has a recognized role in metastasis and tumor invasiveness.[12,13] 67LR is also known to facilitate attachment and migration of endothelial cells and, given its positive correlation with microves-

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sel density in tumors, endothelial cell-associated 67LR is also likely to have a crucial function in tumor angiogenesis.[13–15] In a murine model of proliferative retinopathy, 67LR was found to be highly expressed by proliferating intraretinal and preretinal new vessels.[16] This was in direct contrast to the established, quiescent, retinal vasculature where expression was barely detectable.[16] In addition, it has been shown that 67LR is highly expressed by proliferating microvascular endothelium during retinal development[17] and that, *in vitro*, expression levels decrease significantly when these cells become contact-inhibited.[9]

It has been shown that synthetic peptides with homology to the binding site of 67LR on the laminin β1 chain (residues 925–933 of murine β1; sequence CPDGYIGSR) may display agonist or antagonist properties and can effectively enhance or reduce epidermal growth factor (EGF)- or laminin-stimulated endothelial cell motility, respectively.[10] For example, a peptide antagonist of 67LR, derived from the murine EGF amino acid sequence 33–42 ($EGF_{(33-42)}$; sequence VIGYSGDR) inhibits endothelial cell motility *in vitro*, whereas the native laminin β1 peptide ($Lam\beta1_{925-933}$) acts as an agonist, stimulating endothelial cell motility.[10,14,18] Thus, 67LR represents a potentially useful therapeutic target for modulating retinal neovascularization and to test this we used synthetic peptides which display either agonist or antagonist properties in a murine model of hypoxia-induced proliferative retinopathy.

Materials and Methods

Synthesis of Peptide Analogues

The following synthetic peptides were used in the experiments: a decapeptide from the C-loop of murine EGF (EGF_{33-42}) (acetyl-C-(S-Acm)-VIGYSGDR-C(S-Acm)-NH_2), a scrambled, control peptide (based on a randomized peptide from the EGF_{33-42} sequence; acetyl-IDC-(S-Acm)-YGC-(S-Acm)-RSVG-NH_2) and a nonapeptide $Lam\beta1_{925-933}$ (amino acid sequence: CPDGYIGSR), corresponding to the binding region for 67LR on laminin β1 chain (amino acid residues 925–933) ($Lam\beta1_{925-933}$). For fluorescein isothiocyanate (FITC) labeling of EGF_{33-42}, the carboxyfluorescein (Fluka, Dorset, UK) was coupled to the amino-terminal cysteine using fluorenylmethcarbonyl (Fmoc) chemistry. All peptides were synthesized on a model 432Λ peptide synthesiser (Applied Biosystems, Warrington, UK), using standard solid-phase Fmoc procedure. They were then purified using reverse-phase high performance liquid chromatography (HPLC) and the purity confirmed by capillary electrophoresis, automated amino acid analysis, and electrospray mass spectrometry.

Animal Model and Experimental Groups

The studies adhered to the Association for Research in Vision Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research. Oxygen-induced retinopathy (OIR) was induced in C57BL/J6 mice according to a protocol which has been described previously.[19] Briefly, litters of 7-day-old (P7) pups and their nursing dams were exposed to 75% oxygen for 5 days. The flow of humidified medical grade oxygen was controlled by a gas oxygen controller (PROOX model 110; Reming Bioinstruments, Redfield, NY). On postnatal day 12 (P12) the mice were returned to ambient oxygen. Body weights were recorded on P7 and daily from P12 to P20 to ensure that there was no serious growth retardation.

$FITC\text{-}EGF_{33-42}$ was administered intraperitoneally (i.p.) into hypoxia-exposed ($n = 5$) and normoxia control mice ($n = 4$) at P20. At 2, 6, and 24 hours postinjection the eyes were enucleated and fixed in 4% paraformaldehyde (PFA). The anterior segment, lens, vitreous, and hyaloid were removed and the posterior eye cup was subjected to four radial full-thickness cuts and incubated for 16 hours at 4°C in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 (TX-100). The retinal vasculature was then localized through labeling with biotinylated BSII lectin (purified from *Griffinia simplicifolia*, Sigma Chemical Company) and subsequently, streptavidin-Texas Red (Dako Ltd.). The eye cups were then flat mounted and fluorescence was localized using a confocal scanning laser microscope (CSLM). Kidneys, liver, and brain from these animals were also harvested and fixed before frozen sections were prepared and viewed by CSLM.

Non-labeled EGF_{33-42}, and $Lam\beta1_{925-933}$ in two different concentrations (10.0 and 2.0 mg/kg/day, diluted in PBS) were administered i.p. daily from P12 to P19 to a minimum of 12 pups per group. Scrambled peptide was used in the highest dose (10.0 mg/kg/day) only.

Each litter of hypoxia-exposed mice was divided into two groups, one of which was a peptide-treated group and the other was designated to be injected with either PBS or scrambled peptide and was used as the control for each experiment. Both groups stayed together throughout the whole experiment (P0 to P20) to eliminate a possible difference in growth rate originating from different nursing conditions. Routinely, two pups from a litter were killed upon returning to the room air (P12) to check for hypoxia-mediated closure of the central retinal capillaries and the rest of the litter 8 days later (P20). Before sacrifice, the mice were deeply anesthetized by intraperitoneal injection as previously described[16] and given 0.15 ml of FITC-dextran (50 mg/ml in PBS) via the left ventricle (fluorescein isothiocyanate dextran, MW: 2×10^6; Sigma-Aldrich). The eyes were enucleated, fixed in 4% paraformaldehyde solution in 0.1 mol/L phosphate buffer for 18 hours, and then washed in PBS. The retinas were carefully dissected and flat mounted on microscope slides in a Maltese cross configuration.

Quantification of Neovascular Response

Flat-mounted FITC-dextran perfused retinas were imaged on a BioRad MicroRadiance confocal scanning laser microscope fitted to an Olympus BX60 fluorescence microscope with a 4× plan-apochromatic objective. For

comparative analysis, the retinal angiographic images were always orientated with the optic nerve at the center of the field of view. The angiographic analysis was conducted according to a novel method (Gebarowska D, Stitt AW, Mahon A, Nelson J, Gardiner TA, submitted for publication) which displayed each digital angiographic image with a superimposed 64-square grid (8 × 8 squares) corresponding to a real area of 9.95 mm^2. Each grid square, equivalent to 0.155 mm^2 of retinal area, was analyzed and annotated with on-screen letters which specifically recorded and quantified normally vascularized retina, residual ischemic retina at P20, and the vasoproliferative response as registered by preretinal neovascularization and intraretinal tufts of new vessels. A computer program using the classification described below was designed to assist with the retinal angiogram analysis and classified vessels according to the following: E, empty; N, normally vascularized retina; I, ischemic non-perfused retina; T, neovascular tufts; O, non-vascularized far periphery; V, tortuous vessels; and U, unidentifiable. "Empty" represented those areas of an image corresponding to the expansion of the four radial cuts applied in the flat-mounting procedure. An operator familiar with the relevant angiographic morphology applied the letter codes. The annotation procedure allowed for the recording of different features within any given grid square, ie, coding a square by more than one letter, which was usually necessary as a retinal area of 0.155 mm^2 may be characterized by several angiographic features. The program was able to quantify all possible letter combinations and calculate the total retinal areas displaying the particular morphologies. The total areas characterized by each of the designated features were expressed in mm^2 and as percentages of a total analyzed retinal area. The program performed simple summary statistics of an analyzed image and the data files were transferred to other programs for further analysis and display where the data were compared by one-way analysis of variance.

Since litter size was variable and there was always control and experimental animals within litters, the number of mice in each experiment was variable. However, for all experiments there was a minimum of 12 eyes analyzed from 6 mice and a maximum of 24 eyes from 12 mice per group.

Animal Growth, Organ Weights, and Histological Analysis

Mice body weights were recorded on P7 and daily from P12 to P20 and compared between two groups of the same "nursery" litter. At the end of the experiments hearts, livers, lungs, kidneys and spleens were collected, weighed, fixed in 4% paraformaldehyde, and processed for light microscopic examination after hematoxylin and eosin staining. An experienced pathologist evaluated the organs for alterations in morphology.

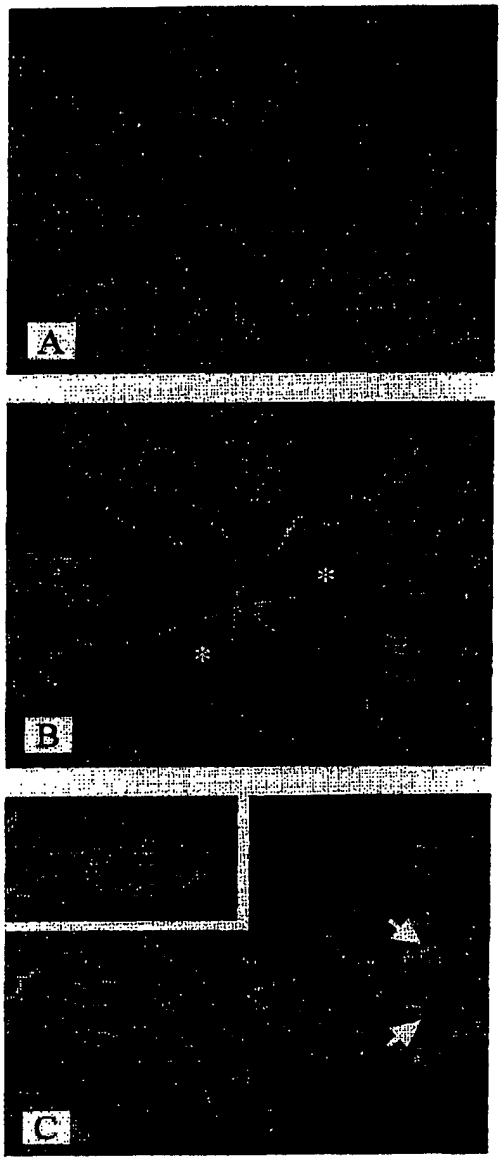

Figure 1. Hypoxia-induced proliferative retinopathy in the mouse model. A: Normal murine retinal vasculature showing perfusion of central retina as assessed by confocal scanning laser microscopy of angiograms (Magnification, ×40). Following exposure of hypoxia (75% for 5 days between P7 and P12) there is closure of the central capillary beds (B) which is followed by preretinal neovascularization up to P21 in response to retinal hypoxia in normal oxygen conditions (C; **inset**, ×200 magnification of neovascular frond). **Arrows** depict preretinal neovascularization; * indicates avascular regions at P12. Original magnification, ×40.

Results

In comparison to retinas from mice which had not been pre-exposed to hypoxia, experimental mice consistently exhibited closure of the central retinal vasculature at P12 (compare Figure 1,A and B). By P20 there was evidence

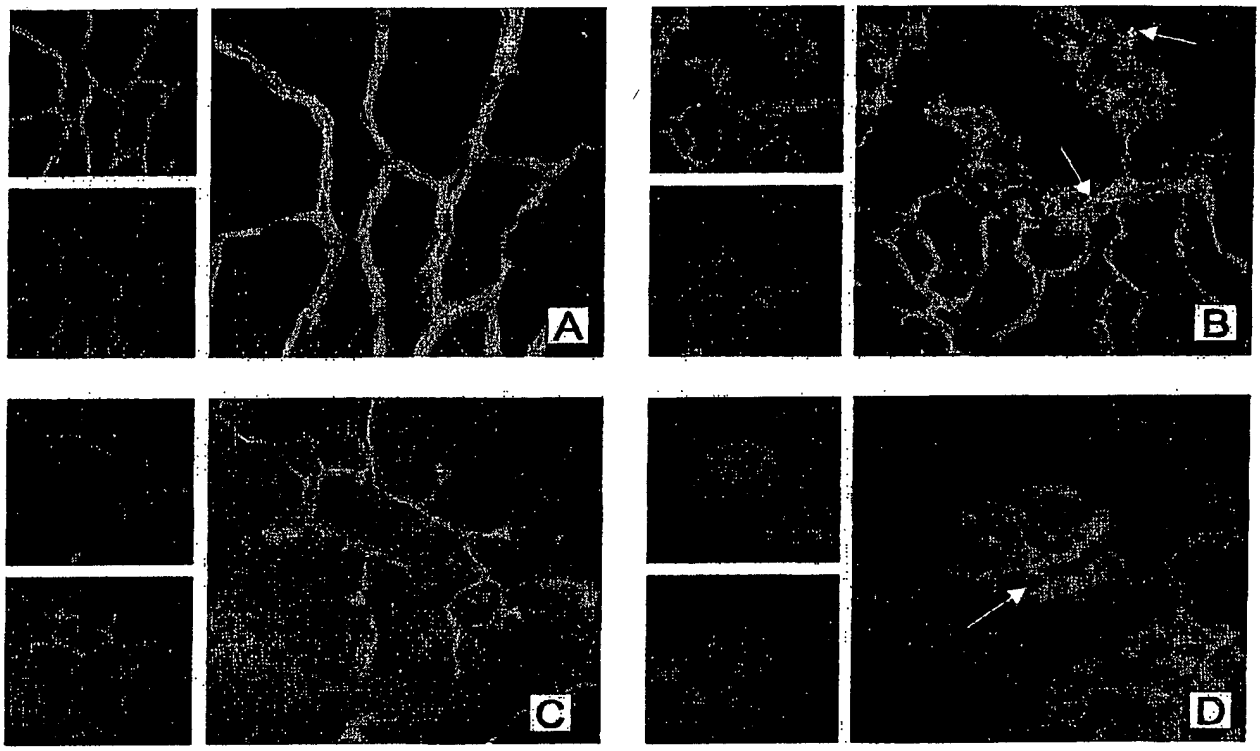

Figure 2. Localization of FITC-labeled EGF_{33-42} in the murine retina. Throughout this figure the red channel image is lectin staining; green channel image is FITC-EGF_{33-42} and the largest image is a red/green merged image. **A:** In normoxia-exposed control mice (P20) i.p.-injected FITC-EGF_{33-42} was localized to the retinal vasculature of the nerve fiber layer within 2 hours. There was evidence that the peptide had tranversed the blood retinal barrier as depicted in green fluorescence within the cytoplasm of the ganglion cells. Original magnification, ×800. **B:** In hypoxia-exposed P20 mice the FITC-EGF_{33-42} was localized to the intra and preretinal vessels 2 hours after injection. Hyperfluorescent areas may indicate an accumulation of the peptide within the neovascular fronds (**arrows**). Original magnification, ×400. **C:** Within the inner plexiform layer the green fluorescence of the peptide analogue was largely localized to the capillaries of the deep capillary plexus although there was also evidence of some leakage into the neural retina. Original magnification, ×400. **D:** After 24 hours postinjection of FITC-EGF_{33-42} there was still green fluorescence in the neovascular fronds (**arrow**) although this was less widespread in the surrounding vasculature than after 2 hours. There was still diffuse fluorescence in the neural retina. Original magnification, ×600.

of a proliferative response after return to normoxic conditions, with fronds of new retinal vessels being visible in the fluorescein angiograms (Figure 1C).

CSLM analysis 2 hours after FITC-EGF_{33-42} injection of P20 control mice demonstrated that this peptide reached the retinal vasculature and had apparently crossed the blood-retina barrier (Figure 2A). When labeled peptide was introduced into hypoxia-exposed mice there were intense accumulations of this labeled peptide within the neovascular fronds at 2 and 6 hours (Figure 2B). FITC was also delineated to the superficial and deep capillary beds (Figure 2C). At 24 hours postinjection there was considerably less FITC label in the preretinal vasculature and it appeared to be largely basement membrane associated in the intraretinal vessels (Figure 2D). In sections of other organs FITC-EGF_{33-42} was apparent in the profiles of blood vessels with diffuse fluorescence apparent in the extravascular space. In kidney there was some evidence of diffuse fluorescence in the tubule epithelium (data not shown).

Evaluation of the fluorescein angiograms coupled with a computerized analysis approach allowed quantification of the absolute areas of normally vascularized retina, avascular tissue, and areas of preretinal neovascularization. It was found that the retina of P12 mice with OIR showed total non-perfusion in the regions of the central retinal capillary beds, estimated at a mean area of 5.43 ± 0.46 mm^2 (corresponding to 66.5 ± 6.2% of the total retinal area). The retina of hypoxia-treated P20 mic showed a significant preretinal neovascular response, which extended from the surviving peripheral vasculature, and variably from the optic disk to cover previously ischemic central retina (Figure 1C). The mean area of retina showing preretinal neovascularization in these animals measured 4.94 ± 0.61 mm^2 (equivalent to 59.1 ± 7.3% of the total retinal area).

Evaluation of retinas from mice treated from P12 with $mEGF_{33-42}$ (Figure 3A) showed a clear inhibition of neovascularization compared with a scrambled peptide-injected control group (Figure 3B). At the same time, EGF_{33-42} treatment had no effect on quiescent, nonproliferative retinal vessel density. On quantification, there was a significant reduction in the occurrence of preretinal vessels in mice treated with $EGF_{(33-42)}$ at either 2.0 ($P < 0.005$) or 10.0 mg/kg/day ($P < 0.001$) compared with controls (Figure 3C). The lowest concentration

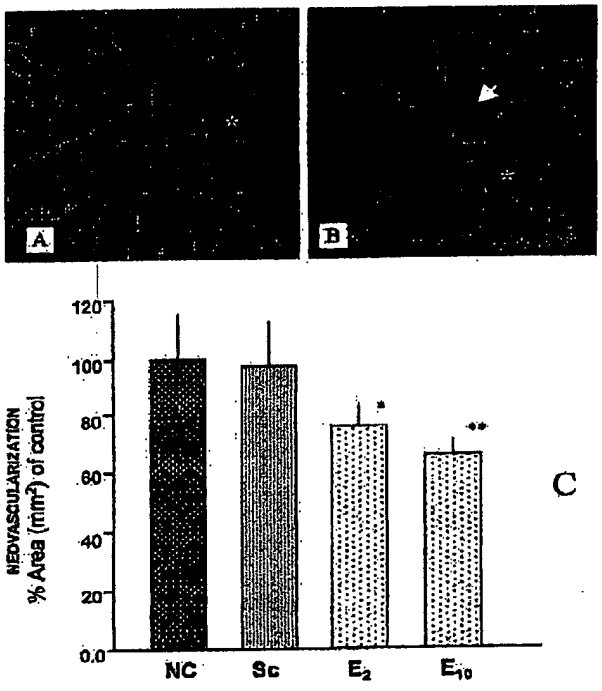

Figure 3. Modulation of proliferative retinopathy by the 67LR peptide antagonist EGF_{33-42}. Treatment of mice with EGF_{33-42} (daily from P12 to P20; 2 or 10 mg/kg, i.p.) caused a significant reduction in neovascularization above the internal limiting membrane (A; compare with Figure 2B). Treatment with a scrambled peptide sequence lead to no significant reduction in preretinal neovascularization (B; compare with Figure 1C). Original magnification, ×40. C: Treatment of mice with EGF_{33-42} at 2 or 10 mg/kg/day (E2; E10) resulted in a significant reduction in neovascularization when compared to non-treated control (NC) or scrambled peptide (SC) groups (n>12 mice/group) (* $P < 0.005$; ** $P < 0.001$). **Bars** show SEM.

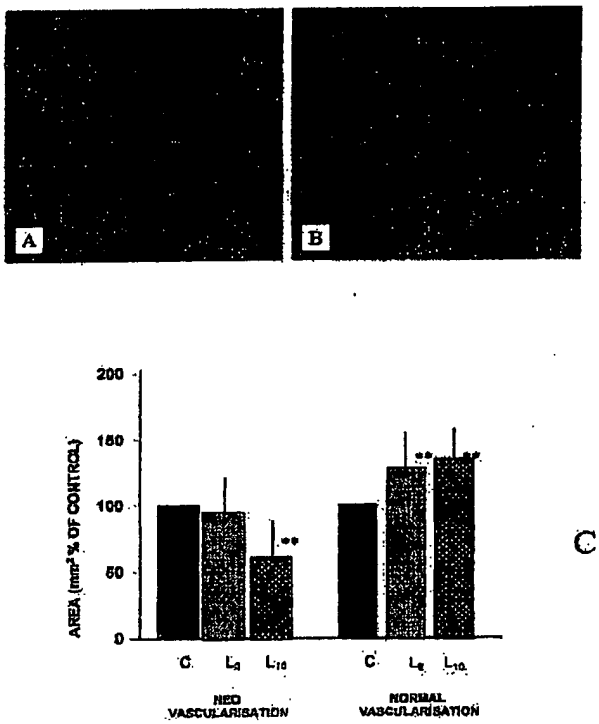

Figure 4. Treatment with the 67LR agonist $Lamb1_{925-933}$ modulates retinal ischemia and neovascularization. Similarly to EGF_{33-42} (Figure 3), $Lam\beta1_{925\ 933}$ also caused a reduced neovascularization at 2 mg/kg/day (A) and 10 mg/kg/day (B). However, there was a concomitant increase in intraretinal revascularization and increase in retinal vascular "normality" (compare Figure 4, A and B with Figure 3, A and B). Original magnification, ×40. C: $Lam\beta1_{925-933}$ at the highest dose produced a reduction in neovascularization comparable to that of EGF_{33-42} (** $P < 0.001$). There was a significant increase in intraretinal revascularization of the central retina with accompanying reduction in ischemia (** $P < 0.001$). Generally there was a tendency toward "normality" when compared to hypoxia-exposed scrambled peptide-treated control (C) mice. **Bars** show SEM.

proved to be equally effective at reducing preretinal neovascularization in hypoxia-exposed mice at P20.

P20 mice which had been treated with $Lam\beta1_{925-933}$ for 8 days demonstrated a significant reduction in retinal neovascularization at the highest dose of 10 mg/kg/day ($P < 0.001$ when compared to control, hypoxia-exposed mice) (Figure 4). Full angiographic analysis of this series of experiments revealed marked differences compared with the appearance of EGF_{33-42}-treated retinas. In $Lam\beta1_{925\ 933}$-treated animals there was a marked increase in the central retinal capillary density in comparison to EGF_{33-42} and control retinas (compare Figure 4, A and B with Figure 1C and Figure 3A). Quantitative analysis revealed a significant reduction in retinal ischemia and an increase in intraretinal revascularization of the ischemic central retina ($P < 0.001$) with a tendency toward "normality" when compared to scrambled peptide-treated mice or hypoxia controls (Figure 4C).

During treatments, neither EGF_{33-42}, $Lam\beta1_{925-933}$, or the scrambled peptide had a significant influence on mouse body weight when compared to hypoxia-treated control mice. On postmortem examination of other organs it was apparent that the peptides also did not influ nce weights of liver, lungs, or spleen. However, with EGF_{33-42} treatments there was a significant increase in heart weight ($P < 0.033$) and decrease in kidney weight ($P < 0.038$) when compared to controls. On pathological assessment of the organs there was no apparent influenc of any of the peptide treatments on tissue organization or qualitative vessel density (data not shown).

Discussion

A previous study by Stitt et al[16] has shown that 67LH is up-regulated in proliferating retinal vasculature during hypoxia-induced proliferative retinopathy. Furthermore, expression of this receptor is also increased during murine postnatal retinal vascular development.[17] Following on from this, the present investigation has demonstrated that the 67LR antagonist EGF_{33-42} can achieve high concentrations in the retinal vasculature when injected i.p. and such treatment significantly inhibits preretinal neovascularization in the murine model of OIR. It is apparent, therefore, that this laminin receptor plays a critical role in proliferative retinopathy, probably by proventing endothelial cell migration which is an essential component of

the angiogenic process. This is supported by *in vitro* studies using $EGF_{(33-42)}$ which showed that this peptide significant inhibits 67LR-mediated endothelial cell motility and migratory capacity.[10] Most proliferating cells require the expression of adhesion molecule receptors to interact with their underlying substratum.[13] 67LR, by virtue of its high affinity for laminin, appears to have an important role in rapidly anchoring cells to the substratum during cell proliferation before integrin-mediated cell spreading.[15,20]

It has been shown that once proliferation responses reduce (eg, as a result of contact inhibition *in vitro*), endothelial cells, including retinal microvascular endothelium, markedly down-regulate their expression of 67LR.[9,16] Indeed, it has been shown that 67LR expression is reduced or absent on quiescent intraretinal capillaries in comparison to actively proliferating vessels.[16] In the current study, EGF_{33-42} had no apparent influence on quiescent, non-proliferative retinal vessel density as evidenced by the persistence of the central retinal ischemia and no change at the peripheral capillary plexi or large central retinal vessels. Being a cell surface receptor which is comparatively highly expressed by proliferating endothelial cells makes 67LR an promising target for treatment of retinal neovascularization and perhaps other angiogenic diseases such as metastatic cancer.

That $Lam\beta1_{925-933}$ caused a reduction in neovascularization comparable to that of EGF_{33-42} was unexpected, as this peptide has been previously shown to function as an agonist of 67LR-mediated endothelial cell migration *in vitro*.[10] However full angiographic analysis revealed differences compared with the appearance of EGF_{33-42}-treated retinas in that there was a clear tendency toward "normality" when compared to hypoxia-exposed control mice. The data suggests that in this model system the $Lam\beta1_{925-933}$ nonapeptide is acting as a partial 67LR agonist, in that it promotes endothelial cell migration and revascularization of the ischemic neural retina. Significantly, there was also a decrease in preretinal neovascularization which is most likely due to the reduction in retinal ischemia and hence hypoxia-mediated expression of potent angiogenic stimuli such as VEGF.

This is the first report on the ability of a fragment of the β chain of laminin-1 to cause a significant revascularization event in any tissue. However, previous studies have suggested that peptide fragments based around the YIGSR motif can act as 67LR agonists and promote endothelial cell sprouting, adhesion, and tube formation *in vitro*.[14,21] The present results resemble those obtained from studies of the bioactivity of the peptide SIKVAV (a motif derived from the α chain of laminin-1) which can also promote angiogenesis *in vitro* and *in vivo*[22,23] and can appreciably reverse hind limb ischemia by augmenting capillary recanalization.[24] It is possible that agonist stimulation of the retinal vasculature with $Lam\beta1_{925-933}$-based peptides may allow more rapid cell spreading and thus enhance the endothelial angiogenic phenotype.

Reversal of retinal ischemia would be a highly effective therapeutic option for many important retinal disorders such as diabetic retinopathy and retinopathy of prematurity. Agonism of 67LR and other similar receptors may lead to a reversal of retinal vascular insufficiency through promotion of intraretinal angiogenic activity without leading to uncontrolled proliferation on the retinal surface, a phenomenon which carries high risk of vitreal bleeding and tractional retinal detachment. In many disease states, a strategy of ischemia reversal has obvious advantages over agents which directly inhibit endothelial cell proliferation since it offers the option to repair tissue damage which is the primary stimulus for neovascularization. The potential uses of such pro-angiogenic peptides warrants further study.

Acknowledgment

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ARTICLES

Inhibition of angiogenesis and tumor growth by a synthetic laminin peptide, CDPGYIGSR-NH2

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A laminin-derived synthetic peptide, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH2 (CDPGYIGSR-H2), containing an active site for cell binding inhibited both angiogenesis and solid tumor growth. It potently suppressed both embryonic angiogenesis of the chick chorioallantoic membrane and migration of vascular endothelial cells induced by a tumor-conditioned medium but neither the in vitro proliferation of endothelial cells nor that of tumor cells. Additionally, in in vivo tests, CDPGYIGSR-NH2 markedly inhibited both the growth of s.c. solid tumor of Sarcoma 180 and that of Lewis lung carcinoma (3LL) in the lungs. On the contrary, ascitic tumor growth of Sarcoma 180 was not affected by this peptide, even though the same cell source was used. It was concluded that solid tumor growth inhibition by CDPGYIGSR-NH2 was due not a direct effect on cell growth but to antiangiogenic effect mediated by the inhibition of endothelial cell migration.

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ARTICLES

A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice

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The mechanisms by which tumor cells metastasize to bone are not well understood. We have investigated the role of the basement membrane glycoprotein, laminin, in bone metastasis, since antagonists to laminin have been shown to inhibit the formation of lung metastases. We studied the formation of osteolytic metastases caused by a human tumor which is known to cause osteolysis and hypercalcemia in nude mice. We found that tumor-bearing nude mice developed hypercalcemia, cachexia, and characteristic osteolytic lesions throughout the skeleton after injection of this human melanoma cell line (A375) into the left ventricle. When we gave injections to nude mice with A375 cells which had been exposed to C(YIGSR)3-NH2, a laminin-derived synthetic peptide containing three linear sequences of YIGSR with an amino-terminal cysteine which competes with laminin for its receptor, we found a decrease in the formation of detectable osteolytic bone metastases. The tumor cells were incubated with the antagonist and then inoculated into nude mice which were administered the antagonist i.p. Hypercalcemia and cachexia were also decreased in tumor-bearing mice treated with the laminin antagonist. In contrast, laminin itself increased the number of osteolytic bone metastases, as has been shown for other tumor cells. These data suggest that laminin plays a role in the formation of osteolytic bone metastases in this model and that laminin antagonists may be useful in the prevention of bone metastases in some human tumors.

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and reducing agents, EDTA-Hin(139–190) cleaves double-stranded DNA at the recognition sequence of the Hin recombinase site, revealing the location of the NH_2-terminus of Hin(139–190). The coupling of a DNA-binding peptide to a metal chelator creates a hybrid peptide capable of cleaving specific sites on DNA. Design of other multifunctional peptides capable of recognizing specific substrates and chemical modification of those substrates could lead to reagents for use in chemistry, molecular biology, and medicine.

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YIGSR, a Synthetic Laminin Pentapeptide, Inhibits Experimental Metastasis Formation

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The invasion of tumor cells through basement membranes is a critical step in the formation of metastases. The binding of the malignant cells to laminin in the basement membranes allows their attachment and activates their invasiveness. Recently a synthetic nonapeptide from the B1 chain sequence of laminin was identified as a major site for cell binding. A pentapeptide within the nonapeptide sequence was found to reduce the formation of lung colonies in mice injected with melanoma cells and also to inhibit the invasiveness of the cells in vitro.

LAMININ, A BASEMENT MEMBRANE–specific glycoprotein, has various biological activities including promoting the attachment, growth, and differentiation of epithelial cells (*1*). It also appears to be involved in tumor cell invasion and metastasis. Malignant cells have more laminin on their surface, bind more laminin, and attach more readily to laminin (*2, 3*). Laminin increases their invasive and metastatic activity (*3, 4*) and induces the secretion of collagenase IV (*5*). These activities appear to involve the binding of laminin to a high affinity receptor on the cell surface (M_r = 67,000) (*6*), since proteolytic fragments of laminin ($M_r \cong$ 450,000) that bind to the receptor and block the formation of metastases (*4, 7*).

Laminin is composed of three chains, A(400 kD), B1(230 kD), and B2(220 kD) chain (*8*), which are arranged in a cross-shaped structure (Fig. 1). We have cloned and sequenced the B1 chain (*9*), prepared synthetic peptides and peptide-specific antibodies, and used these to identify a sequence (CDPGYIGSR) (*10*) in the B1 chain which is active in cell attachment, chemotaxis, and binding to the laminin receptor (*11*). In this report, we have tested the nonapeptide (called peptide 11) and its amide form (peptide 11–amide) as well as other peptides in (i) an in vitro invasion assay (*12*) and (ii) a murine model of lung tumor colonization after the intravenous injection of B16F10 melanoma cells (*13*). The in vitro invasion assay measures the ability of cells to attach, degrade, and migrate through a reconstituted basement membrane matrix. By means of this assay, invasiveness has been found to strongly correlate with metastatic activity (*12*). We find that peptide 11 and its terminal pentapeptide YIGSR inhibit tumor cell invasion. In other studies on cell adhesion and receptor binding, we found that peptide 11–amide was more active, probably because it neutralized the negative charge on the arginine (*14*). The amide form also appeared to be more active in the in vitro invasion assay (Fig. 2). Other peptides of 19 to 22 amino acids in length from different domains in the B1 chain of laminin (peptides 1-7 in Fig. 1) were inactive (Fig. 2; P2 is shown). Similar findings on the activity of the peptides were obtained in the in vivo assay for lung tumor colonization (Fig. 3). Both peptide 11 and peptide 11–amide reduced the numbers of lung tumors (by 74% and >90%, respectively) when administered with B16F10 cells by tail vein injection into mice (Fig. 3). The inhibition of colonization

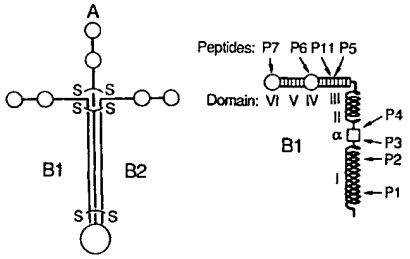

Fig. 1. Schematic model for the B1 chain of laminin. Seven structural domains in the B1 chain of laminin have been described and these are designated I–VI and α (*9*). The circles designate the globular regions of the laminin and the square designates an unusual cysteine-rich homologous repeat. P1 (residue 1593–1611), KQADEDIQGTQNLLTSIES; P2 (residue 1509–1529), KSGNASTPQQLQNLTEDIRER; P3 (residue 1395–1416), CRTDEGEKKCGGPGCGGLVTVA; P4 (residue 1363–1383), KLQSLDLSAAAQMTCGTPPGA; P5 (residue 960–978), NIDTTDPEACDKDTGRCLK; P6 (residue 615–634), KIPASSRCGNTVPDDDNQVV; P7 (residue 364–385), PERDIRDPNLCEPCTCDPAGSE; P11 (residue 925–933), CDPGYIGSR. Peptides were synthesized with an automated synthesizer, model 430 A (Applied Biosystems, Inc., Foster City, California). Their purity was checked by amino acid analyses and by high performance liquid chromatography.

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by the nonapeptide was dose-dependent (Fig. 4); lower doses (50 to 100 μg per mouse) inhibited the formation of the majority of the colonies in the lung but much higher doses (1 mg per mouse) were required for total blockade. To better define the active sequence, various peptides were tested at the high concentration (1 mg per mouse) for their ability to prevent colonization in the lung (Fig. 5). A peptide, NIDTTDPEACDKDTGRCLK, from another domain in laminin did not show any activity. CDPGYIGSR-NH_2 showed the greater inhibition of lung tumor colony formation (more than 90%). When the nonapeptide and cells were injected into separate tail veins, a similar degree of inhibition of lung tumors was observed, demonstrating that preincubation of the cells with the peptide is not required. The pentapeptide YIGSR and its amide form showed a comparable degree of inhibition. The amide form of the reverse sequence, RSGIY-NH_2, showed much lower activity. Pentapeptide YIGSE was less active, suggesting that arginine is important for activity. RGDS-NH_2 was also tested because GRGDS, a sequence from the cell-binding domain of fibronectin, has been reported to inhibit lung colonization by melanoma cells (*15*). This peptide reduced lung colonies by about 30% and RGDSGYIGSR-NH_2, which contains both the RGDS and the GYIGSR-NH_2 sequences, inhibited lung tumor colonization by about 50%. These data suggest that YIGSR from the B1 chain of laminin is more active than the fibronectin-derived sequences in inhibiting lung tumor colonization; the reason for this may be the higher affinity of laminin than fibronectin for receptor binding (*16*).

The inhibition of lung tumor colonization by the active peptides was not due to cytotoxicity since incubation of cells with peptide 11 or peptide 11-amide at 10 mg/ml for 20 minutes did not affect their subsequent proliferation rate or the final cell density attained on culture dishes (*17*). Furthermore, these peptides did not inhibit lung tumor colonization by altering the tumorigenicity of the cells. The weight of tumors which formed 14 days after melanoma cells were injected subcutaneously with or without 1 mg of these peptides were similar [CDPGYIGSR (mean ± SD = 1.2 ± 0.7 g, n = 6), its amide form (1.3 ± 0.7 g, n = 6), and control (1.0 ± 0.6 g, n = 6)]. Lastly, preincubation of the melanoma cells with the peptides did not show any effect on cell aggregation.

As there was an exact correlation between the inhibitory activities of the various peptides in the in vitro and in vivo assays, it seems likely that YIGSR inhibits lung tumor colony formation by blocking tumor cell invasion through basement membranes. In agreement with this conclusion is our finding that YIGSR is active in the attachment of fibrosarcoma HT-1080, Chinese hamster ovary (CHO) cells (*11*), and B16F10 cells (*18*). In addition, the pentapeptide elutes the 67-kD laminin cell surface receptor from a laminin affinity column (*11*). We have also directly tested this peptide on cellular activities implicated in invasion. YIGSR inhibits cell adhesion (60% at 100 μg/ml) and migration toward laminin (80% at 200 μg/ml), but has no effect on collagenase production at levels up to 300 μg/ml; 10 μg/ml of

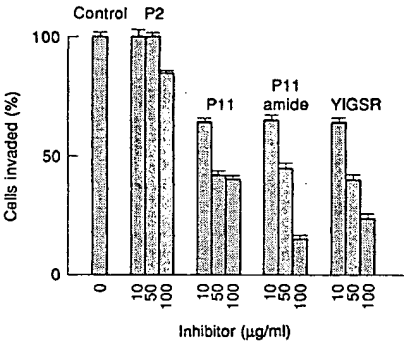

Fig. 2. Inhibition of B16F10 melanoma cell invasion in vitro by synthetic peptides of the B1 chain of laminin. B16F10 melanoma cells were obtained from I. J. Fidler, Houston, Texas, and were propagated under standard culture conditions. The chemoinvasion assay was carried out as described (*12*). Briefly, polycarbonate filters (8-μm pore size; Nuclepore, Pleasanton, California) were coated with 50 μg of a reconstituted basement membrane (*12*) and placed in a Boyden blind well chemotaxis chamber. Conditioned medium (0.2 ml), obtained by incubating NIH 3T3 cells for 24 hours in serum-free medium, was placed in the lower compartment of the Boyden chamber. B16F10 melanoma cells were detached by the addition of 0.02% EDTA, suspended in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum, washed with DMEM containing 0.1% bovine serum albumin, and resuspended in this latter medium. Cells (3×10^5 per 0.8 ml) were placed in the upper compartment of the Boyden chamber with the peptides being tested. The concentrations of peptides in the upper compartment were 10, 50, and 100 μg/ml. After incubation for 5 hours at 37°C in 5% CO_2 and 95% air, the filters were fixed with methanol, stained with hematoxylin and eosin, and the cells migrating through the basement membrane were counted. In the absence of peptides, 25 cells migrated per field. Each sample was assayed in quadruplicate and the cells in at least five microscopic fields per filter were counted. Bar represents standard error of the mean.

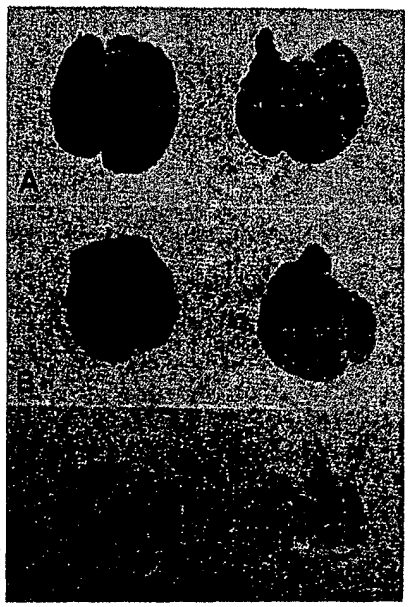

Fig. 3. Effect of peptide 11 and peptide 11–amide on lung tumor colonization. The lung tumor colonization assay was carried out as described (*13*). Peptide 11 (CDPGYIGSR) and peptide 11–amide were dissolved at 10 mg/ml in phosphate-buffered saline (PBS) [0.02M Na_2HPO_4 pH 7.4 and 0.15M NaCl] and were filter-sterilized. The suspension of B16F10 cells (5×10^5) in 0.1 ml of DMEM was mixed with 0.1 ml of the peptide, incubated for 5 minutes at room temperature, and then injected into the tail vein of syngeneic C57BL/6 female mice at 6 weeks of age. Each treatment and control group consisted of eight mice. Three weeks after the injections, the mice were killed and the number of pulmonary tumors on the surface of the lungs was counted. (**A**) Control lungs; (**B**) lungs from animals treated with 1 mg of peptide 11; (**C**) lungs from animals treated with 1 mg of peptide 11–amide.

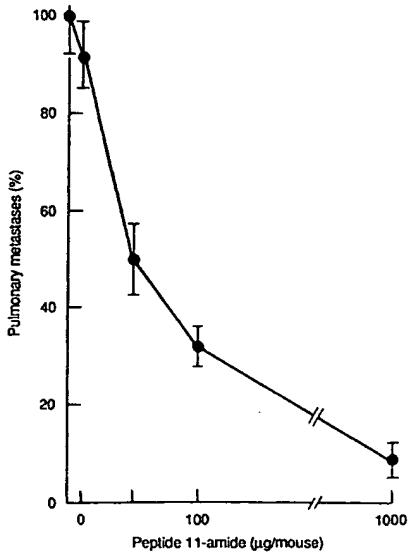

Fig. 4. Effect of peptide 11–amide on inhibition of lung tumor colonies. The assay was carried out as described (Fig. 3) except that various amounts of peptide (0.01 to 1 mg) were mixed with the cells and incubated for 5 minutes before injection. The average number of lung tumors in control mice (tumor cells plus PBS) was 51. Bar represents standard error of the mean (n = 8).

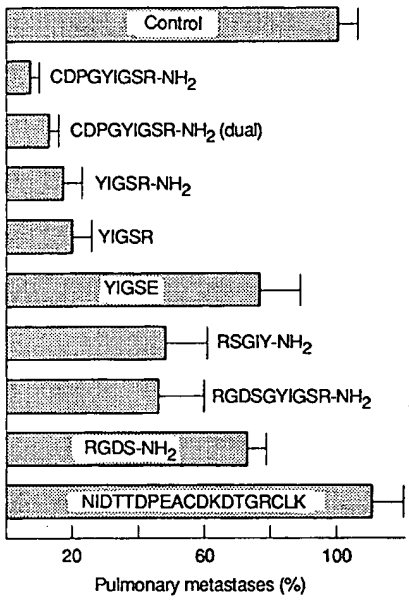

Fig. 5. Inhibitory effect of synthetic peptides on the formation of lung tumors. Peptides were solubilized at either 2 mg/ml (YIGSR) or 10 mg/ml (other peptides) in PBS and injected into mice as described (Fig. 3). Control mice received the same amount of cells and PBS without the peptide. In this group given dual injections of cells plus CDPGYIGSR-NH_2, mice were injected sequentially with melanoma cells via one tail vein and the peptide via the other. Each treatment and control group consisted of eight mice. Two weeks after the injection, mice were killed, lungs were removed, and the number of lung tumors was counted visually. At the time of autopsy, no extrapulmonary tumors were found. In the control mice (lacking the peptide), the average number of tumors was 60. Bar represents standard error of the mean.

laminin caused a fourfold increase in collagenase production (*18*). Taken together, we speculate that YIGSR may inhibit lung tumor colony formation by competing with laminin for the laminin receptor on tumor cells, thus blocking the binding of the cells to basement membranes. The fibronectin/vitronectin cell attachment peptide GRGDS also shows inhibitory activity in tumor cell colonization (*15*). The YIGSR sequence is specific to laminin whereas the RGDS sequence is present in over a hundred proteins (*20*). Since cells interact with both of these proteins via separate and specific receptors, it is likely that these peptides block tumor cell colonization by different mechanisms.

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Two Pairs of Recombination Signals Are Sufficient to Cause Immunoglobulin V-(D)-J Joining

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The minimum sequence requirements for antigen receptor V-(D)-J joining were studied by constructing recombination-substrates containing synthetic recombination signals and introducing them into a recombination-competent pre-B cell line. Two sets of heptamer (CACTGTG) and nonamer (GGTTTTTGT) sequences were shown to be sufficient to cause the V-(D)-J joining, if the 12– and 23–base pair spacer rule is satisfied. A point mutation in the heptamer sequence, or a change in the combination of the two spacer lengths, drastically reduced the recombination.

COMPLETE IMMUNOGLOBULIN (IG) and T cell receptor (TCR) variable region genes are generated by a somatic DNA rearrangement process, which assembles variable (V), diversity (D), and joining (J) gene segments during the differentiation of lymphocytes (*1–5*). Two blocks of sequences, a heptamer CACTGTG and a nonamer GGTTTTTGT are highly conserved adjacent to the germline V, D, and J segments (*6, 7*). The joining takes place between two pairs of recombination signal sequences (RSS's); one pair is separated by a 12–base pair (bp) spacer and the other by a 23-bp spacer (*8, 9*). Three approaches have been taken to study the molecular mechanism of V-(D)-J joining. One approach is the sequence analysis of the recombination region in the V, D, and J segments. Both Ig and TCR genes were shown to contain the heptamer and nonamer sequences separated by a spacer of constant length (Table 1). The second approach is the biochemical characterization of the enzymatic machinery responsible for V-(D)-J joining. It is assumed that at least three activities are needed in the V-(D)-J joining reaction: a DNA binding activity, an endonucleolytic activity, and ligase activity. Candidates for the endonucleolytic activity (*10–13*) were identified in pre-B cells. The third approach is the introduction of artificial recombination substrates with appropriate selectable markers into recombination-competent pre-B cells. Alt and Baltimore and their colleagues (*14, 15*) demonstrated that V-(D)-J joining could take place on exogenous genes. Lewis *et al.* (*14, 16*) identified V-J joining by DNA inversion between the exogenous V_κ and J_κ genes with a retroviral vector system (*17*). Blackwell and Alt (*15*) demonstrated that Ig heavy chain D-J joining occurred by DNA deletion on a plasmid vector introduced by DNA transformation. Yancopoulos *et al.* (*18*) found that even transfected TCR genes could rearrange in the pre-B line 38B9 (*15, 19*).

To define the minimum sequence requirements for the V-J type of recombination, we synthesized two oligonucleotides containing a pair of recombination signals, a heptamer and a nonamer; one pair was separated by a

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